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(54) Title: HUMAN BONE DERIVED INSULIN LIKE GROWTH FACTOR BINDING PROTEIN

(57) Abstract

Purified and isolated compositions of a binding protein for insulin-like growth factors (IGFs) I and II are provided. The binding protein, known as human bone derived IGF binding protein (hBD-IGFBP), potentiates the proliferative effects of IGF-II upon bone cells. Diagnostic assays are also provided for hBD-IGFBP, as well as pharmaceutical compositions and methods for treatment of bone disorders, wound healing, skin repair, and means for modulating IGF activity in bone.

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HUMAN BONE DERIVED INSULIN LIKE GROWTH FACTOR BINDING PROTEIN

Field of the Invention

The present invention relates to bone metabolism, and more particularly to bone metabolic processes which are mediated by a novel insulin-like growth factor binding protein (IGFBP) isolated from human bone. More specifically, the invention relates to a IGFBP termed human bone derived IGFBP (hBD-IGFBP) which potentiates the effect of insulin-like growth factor-II (IGF-II) on bone cell proliferation.

Background of the Invention

IGF-I and IGF-II, the two most abundant growth factors present in human plasma, constitute a family of polypeptides that resemble proinsulin in structure and have both anabolic and acute insulin-like activities in numerous tissues (Daughaday, et al., Endocrine Rev., 10:68-91 (1989)). Although previous studies using rats and mice emphasized IGF-I as the primary IGF, with IGF-II being a fetal hormone, recent findings have pointed to an important role for IGF-II in adult human bone metabolism. IGF-II has been found to be the most abundant growth factor present in human bone, and is the most abundant growth factor produced by human bone cells. Further, IGF-II is one of the few growth factors which is mitogenic to human bone cells. Also, a recently purified inhibitory IGFBP, termed IGFBP-4, was found to inhibit basal bone cell proliferation by about 40% in serum free conditions, suggesting that endogenous production of IGFs contributed substantially to cell proliferation in the absence of added growth factors. And finally, IGF-II receptor blocking antibodies have been shown to inhibit basal bone cell proliferation, suggesting that IGF-II is a key bone cell growth factor (Mohan, et al., Growth, Genetics and Hormones, 6:1-9 (1990) and Mohan, et al., Clin. Orthopedics & Rel. Res., 263:30-48 (1990)).

Recently it has also become clear that a family of structurally related proteins that specifically bind the IGFs are involved in the modulation of IGF action in different tissues. Four classes of human IGFBPs (designated hIGFBP-1, hIGFBP-2, hIGFBP-3 and hIGFBP-4) have been isolated, and the complete amino acid sequences predicted from the nucleotide sequences of the isolated cDNA clones (See, Mohan et al., Clin. Orthopedics & Rel. Res. 263:30-48 (1990); Baxter et al., Prog. Growth Factor Res. 1: 49-68 (1989); Binkert et al., EMBO J. 8:2497-2502 (1988); Brewer et al., Biochem. Biophys. Res. Comm. 152:1289-1297 (1988); Brinkman et al., EMBO J. 7:2417-2423 (1988); Lee et al., Mol. Endocrinol. 2:404-411 (1988); Wood et al., Mol. Endocrinol. 2:1176-1185 (1988); LaTour et al., Mol. Endocrinol. 4:1806-1814 (1990); and Shimasaki, et al., Mol. Endocrinol. 4:1451-1458 (1990).

IGFBP-1 has been isolated from various sources including amniotic fluid, placental membranes, decidua and HEP G2 hepatoma cells. The N-terminal amino acid sequences of the IGFBP-1 proteins isolated from these different sources have been found to be identical. The cloning and complete sequence of cDNA encoding IGFBP-1 from HEP G2, human uterus and human placental cDNA libraries have been reported. IGFBP-2 has been purified from conditioned medium collected from rat liver cells (BRL-3A) and from Madin-Darby bovine kidney cells. The gene encoding IGFBP-2 has been cloned from BRL-3A and human fetal liver cDNA libraries. IGFBP-3 is found in serum as a 150 kilodalton ternary complex between IGF-I or IGF-II, an acid labile glycoprotein of about 85 kilodaltons, and the IGFBP-3 molecule, which is an acid stable glycoprotein of 53 kilodaltons. IGFBP-3 has been purified to homogeneity from human serum and the cloning and sequencing of the cDNA encoding IGFBP-3 has been reported. IGFBP-4 was originally purified from human bone cell conditioned medium as inhibitory IGFBP and from rat serum. The cloning and sequencing of IGFBP-4 cDNA clone isolated from human bone cell cDNA library and liver cDNA library have been recently reported. In addition to these four classes of IGFBPs, Martin et al., J. Biol. Chem., 265:4124-4130 (1990), Roghani et al., FEBS Lett., 255:253-258 (1989), and

Zapf et al., J. Biol. Chem., 265:14892-14898 (1990), report the partial purification of IGFBP from human cerebrospinal fluid, from culture medium conditioned by AG 2804 transformed fibroblasts and from hypoglycemic serum respectively, which exhibit strong affinity for IGF-II over IGF-I.

Thus the art describes a variety of IGFBPs that are produced by different sources and that exhibit disparate binding properties to IGFs as well as different biological functions. For example, IGFBP-1, IGFBP-3 and IGFBP-4 bind both IGF-I and IGF-II with nearly equal affinity, while IGFBP-2 and IGFBP purified from amniotic fluid, fibroblast cells and human serum bind IGF-II with higher affinity than IGF-I. With regard to functions, IGFBP-1 has been shown to both inhibit and potentiate the proliferative action of IGFBP-1 in choriocarcinoma cells and in human fibroblasts (Elgin, et al., J. Biol. Chem., 84:3254-3258 (1987) and Ritvos, et al., Endocrinology, 122:2150-2157 (1988)). IGFBP-3 has been shown to inhibit or stimulate IGF-I actions depending on culture conditions in fibroblasts (De Mellow, et al., Biochem. Biophys. Res. Comm., 156:199-204 (1988)). In contrast to IGFBP-1 and IGFBP-3, IGFBP-4 has only been shown to inhibit IGF-I and IGF-II actions in bone cells (Mohan, et al., Proc. Natl. Acad. Sci. USA, 86:8338-8342 (1989)). The art also suggests that the production of different IGFBPs are modulated disparately in a tissue specific manner. For example, IGFBP-1 production is modulated by insulin while IGFBP-3 production is modulated by growth hormone (Baxter, et al., Prog. Growth Factor Res., 1:49-68 (1989)). These findings suggest that the multiplicity of different IGFBPs in conjunction with their unique regulation, may cause IGF activities to be modulated in a localized, tissue specific manner.

There remains a need in the art to relate IGF-I and IGF-II functions in bone metabolism via IGFBPs, and thus there is a need to identify the IGFBPs produced by bone cells and present in human bone matrix. Such IGFBPs are likely to be involved in regulating bone metabolism and can be used in clinical assays to provide information in the diagnosis of defects in bone metabolism. IGFBPs which potentiate the IGF-

dependent growth of bone would be particularly useful in therapeutic applications for treatment of metabolic bone diseases such as osteoporosis. Quite surprisingly, the present invention fulfills these and other related needs.

Summary of the Invention

The present invention provides methods and compositions for clinical diagnosis and treatment of metabolic disorders related to IGF-mediated bone formation and cell proliferation. More particularly, the invention provides human bone derived IGF binding protein (hBD-IGFBP). The hBD-IGFBP acts synergistically with IGF-II to potentiate IGF-II mediated cell proliferation under conditions where both IGF-II and IGFBP are administered simultaneously. The purified hBD-IGFBP also binds to hydroxyapatite with strong affinity and thus provides a reagent to target molecules specifically to bone, such as IGF-II and/or IGF-I, other growth factors or drugs which may affect bone resorption or formation, and thus is useful in treatment of, e.g., bone fractures and bone diseases such as osteoporosis or osteosarcoma. hBD-IGFBP may also be used in the treatment of wound healing and in skin repair. hBD-IGFBP may be used diagnostically as a marker of bone formation rate, such as during treatment of bone disorders with therapeutic agents. hBD-IGFBP may also be used as a reagent for clinical evaluation of IGF levels in samples from patients with bone metabolism and other disorders.

Brief Description of the Drawings

Fig. 1. Comparison of N-terminal amino acid sequence of hBD-IGFBP with that of other known IGFBPs. The sequences are aligned to give maximum identity.

Fig. 2. Competitive binding curves of hBD-IGFBP. The sample was assayed for binding protein activity using labeled IGF-II in the presence or absence of unlabeled IGF-I and IGF-II.

Fig. 3. Protein profile (A) and IGFBP activity profile (B) of the human bone extract in the FPLC Mono Q chromatography step. Three 50 ml aliquots of HA bound fraction pools were applied to the IGF-II affinity column. The resulting three affinity bound fractions were pooled and run on the Mono Q anion-exchange column. Protein profile is monitored by absorbance at 280 nm. Two ml, 2 min fractions were collected. Aliquots of the fractions were diluted 10 fold and assayed for binding protein activity. IGFBP activity is expressed in amounts of specifically bound labeled IGF-II.

Fig. 4. Ligand blot analysis of hBD-IGFBP at different stages of purification. 50 µl of the sample were run on SDS-PAGE (3-27% gradient), transferred to nitrocellulose membranes, blotted with labeled IGF-II, and autoradiographed. Lane a, HA bound fraction of human bone extract; Lane b, IGF-II affinity bound fraction; Lane c, Mono Q IGFBP peak A; Lane d, Mono Q IGFBP peak B; Lane e, Mono Q IGFBP peak C and Lane f, Mono Q IGFBP peak D.

Description of the Specific Embodiments

The invention provides purified and isolated human bone derived IGFBP which binds specifically to IGF-II with greater affinity than to IGF-I. The protein may be purified to homogeneity as desired from proteins extracted from, e.g., human bone preparations, human bone cell conditioned medium, or human serum. Substantially pure hBD-IGFBP of at least about 50% is preferred, at least about 70-80% more preferred, and 95-99% or more homogeneity most preferred, particularly for pharmaceutical uses. Once purified, partially or to homogeneity, as desired, the hBD-IGFBP may then be used diagnostically, as an immunogen, therapeutically, etc.

The hBD-IGFBP produced according to the present invention may be purified by hydroxyapatite apatite chromatography followed by affinity chromatography on a column

with IGF-II and finally for additional purity by Mono Q anion exchange chromatography using an FPLC system. Apparent homogeneity of the purified protein is demonstrated by, e.g., its migration as a single band on SDS-PAGE and by the production of a single amino acid sequence upon N-terminal sequence analysis. Affinity chromatography on an antibody column using antibodies specifically directed against hBD-IGFBP can also be used in a purification scheme. Additional purification may be achieved by conventional chemical purification means, such as liquid chromatography, gradient centrifugation, and gel electrophoresis, among others. Methods of protein purification are known in the art (see generally, Scopes, R., Protein Purification, Springer-Verlag, NY (1982), which is incorporated herein by reference) and may be applied to the purification of the hBD-IGFBP described herein.

Specific binding of hBD-IGFBP to IGF-II and IGF-I is demonstrated by a polyethylene glycol precipitation assay. In this aspect the invention provides a purified protein which is useful in structure-function studies of the determinants of IGFs which allow binding to specific receptors as well as to hBD-IGFBP. Further utility of the purified hBD-IGFBP of the invention is disclosed in the descriptions of other aspects of the invention below.

The purified IGFBP of the invention is unique and distinct from all previously identified IGFBPs. Human IGFBP-1, IGFBP-2, IGFBP-3 and IGFBP-4 are characterized by amino acid sequences which have been published and which are distinct from the amino acid sequence of hBD-IGFBP. The N-terminal amino acid sequence reported for the IGFBP purified from cerebrospinal fluid, fibroblast cell conditioned medium and human serum is also distinct from that of hBD-IGFBP.

The homogeneous human IGFBP of the invention (hBD-IGFBP) is characterized by an N-terminal amino acid sequence identical to, or substantially identical to that shown in Fig. 1. For purposes of invention, an N-terminal amino acid sequence substantially identical to that shown in Fig. 1 is understood to mean an amino acid sequence identical to that of Fig. 1 except for the presence of conservative amino acid

substitutions or other amino acid substitutions, insertions or deletions which do not materially affect the binding of the substantially identical protein to IGF-I or IGF-II, or otherwise materially alter its function in the applications set forth below.

To produce hBD-IGFBP by recombinant routes, the gene which encodes the hBD-IGFBP of the present invention is cloned and expressed by insertion in a suitable expression vector which in turn is used to transform or transfect appropriate host cells for expression of recombinant hBD-IGFBP polypeptide. One or more synthetic oligonucleotide probes reflecting at least a portion of the amino terminal sequence of purified hBD-IGFBP, typically from about 14 to about 25 nucleotides are used to screen a human bone cell cDNA library. The positive clones containing the longest insert is sequenced according to standard procedures. The deduced amino acid sequence is compared with the N-terminal amino acid sequence of the purified protein, and the predicted molecular weight and amino acid composition based on the deduced amino acid sequence are compared with those observed for purified hBD-IGFBP. The clone is then used to produce recombinant hBD-IGFBP by using standard procedures, as generally described in, e.g., Sambrook et al., Molecular Cloning, A Laboratory Manual, 1989 Cold Spring Harbor Press, NY, which is incorporated herein by reference.

In another aspect, the invention concerns polypeptides and fragments of hBD-IGFBP. Polypeptides and fragments of hBD-IGFBP may be isolated from recombinant expression systems or may be synthesized by the solid phase method of Merrifield, Fed. Proc. 21:412 (1962), Merrifield, J. Am. Chem. Soc. 85:2149 (1963), or Barany and Merrifield, in The Peptides, vol. 2, pp. 1-284 (1979) Academic Press, NY, each of which are incorporated herein by reference, or by use of an automated peptide synthesizer. By "polypeptides" is meant a sequence of at least about 3 amino acids, typically 6 or more, up to 100-200 amino acids or more, including entire proteins. For example, the portion(s) of hBD-IGFBP protein which bind hydroxyapatite and/or IGF-II may be identified by a variety of methods, such as by treating purified hBD-IGFBP with a protease

or a chemical agent to fragment it and determine which fragment is able to bind to labeled IGF-II or hydroxyapatite. Polypeptides may then be synthesized and used as antigen, to inhibit IGF-II or hydroxyapatite-hBD-IGFBP interaction, etc. It should be understood that as used herein, reference to hBD-IGFBP is meant to include the proteins, polypeptides, and fragments thereof unless the context indicates otherwise.

In another aspect, the invention provides means for regulating aspects of the hydroxyapatite/hBD-IGFBP/IGF-II interaction, and thus treating, therapeutically and/or prophylactically, a disorder which can be linked directly or indirectly to hBD-IGFBP or to its ligands, such as IFG-II. By virtue of having the binding protein of the invention, agonists or antagonists may be identified which stimulate or inhibit the interaction of IGF-II, hydroxyapatite or other ligand with a hBD-IGFBP. With either agonists or antagonists the metabolism and reactivity of cells in response to hBD-IGFBP or IGF-II are controlled, thereby providing a means to abate or in some instances prevent the disease of interest.

Thus, the invention provides screening procedures for identifying agonists or antagonists of events mediated by the ligand/hBD-IGFBP interaction. Such screening assays may employ a wide variety of formats, depending to some extent on which aspect of the ligand/binding protein interaction is targeted. For example, such assays may be designed to identify compounds which bind to the binding protein and thereby block or inhibit interaction with the IGF-II or hydroxyapatite. Other assays can be designed to identify compounds which can substitute for hBD-IGFBP. Yet other assays can be used to identify compounds which inhibit or facilitate the association of IGF to hBD-IGFBP and thereby mediate the cellular response to IGF.

In another aspect the invention provides a protein which binds IGF-II with selective affinity over IGF-I. Fig. 2 shows the competitive binding curve of the hBD-IGFBP [^{125}I]IGF-II as ligand and IGF-I and IGF-II as competitors. To displace 50% of bound [^{125}I]IGF-II from the IGFBP, about 10 ng/ml of IGF-I and 1 ng/ml of IGF-II was needed, indicating that IGF-II was 10 times more potent than IGF-I in displacing tracer. When

[^{125}I]IGF-I was used as tracer, IGF-II was still more potent (4 times) than IGF-I in displacing tracer. These results suggest that the IGFBP binds IGF-II with greater affinity than IGF-I. Typically, the binding affinity of hBD-IGFBP for IGF-II will range from about 10^{-9} M up to about 10^{-12} M or more, and more likely in the range of at least about 10^{-10} to 10^{-11} M, whereas the binding affinity of hBD-IGFBP for IGF-I ranges about 10 times less, i.e., from about 10^{-8} M up to about 10^{-10} M. The selective affinity of hBD-IGFBP for IGF-II over IGF-I could explain why IGF-II is 10-15 times more abundant than IGF-I in human bone.

The invention provides therapeutic and pharmaceutical compositions of hBD-IGFBP which take advantage of hBD-IGFBP's strong binding affinity to hydroxyapatite, which is at least about 10^{-9} M up to about 10^{-11} M or more. The hBD-IGFBP of the invention binds to hydroxyapatite even in the presence of strong denaturing agents, such as 4M guanidine HCl, while purified IGF-II does not bind to hydroxyapatite. Thus, the hBD-IGFBP provides a means or vehicle to fix or target IGF-II in or to bone. IGF-II can be chemically coupled to hBD-IGFBP via conjugation means that will be readily apparent to those of skill in the art, but should not substantially diminish the desired activity of either protein.

The linkage of hBD-IGFBP to another molecule which is to be targeted to bone tissue or cells, such as the IGFs or other molecules as set forth hereinbelow, can be produced by chemical conjugation using well known laboratory procedures, such as by employing cross-linking reagents. By chemically linked is meant that the protein molecules are linked, typically one to another, typically by covalent bonds. A preferred method of conjugation is the formation of at least one covalent bond between the hBD-IGFBP/IGF-II molecules. The linkage may be direct, which includes linkages containing a synthetic linking group, or indirect, by which is meant a link having an intervening moiety, such as a protein or peptide, e.g., plasma albumin, or other spacer molecule. For example, the linkage may be by way of heterobifunctional or homobifunctional cross-linkers, e.g., carbodiimide,

glutaraldehyde, N-succinimidyl 3-(2-pyridydithio) propionate (SPDP) and derivatives, bis-maleimide, 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), cross-linking without exogenous cross-linkers by means of groups reactive with the individual molecules, such as carbohydrate, disulfide, carboxyl or amino groups via oxidation or reduction of the native protein, or treatment with an enzyme or the like. Methods for chemically cross-linking protein molecules are generally known in the art, and a number of hetero- and homobifunctional agents are described in, e.g., U.S. Pat. Nos. 4,355,023, 4,657,853, 4,676,980, 4,925,921, and 4,970,156, and ImmunoTechnology Catalogue and Handbook, Pierce Chemical Co. (1989), each of which is incorporated herein by reference. In general, such cross-linking should not substantially affect the desired function(s) of IGF-II or hBD-IGFBP.

Hybrid, chimeric or fusion protein molecules of IGF-II and hBD-IGFBP or portions thereof can also be prepared by recombinant DNA techniques, as described in, for example, U.S. Patent 4,859,609, and Sambrook et al., supra, incorporated herein by reference.

The deposition of the IGF-II/hBD-IGFBP complex in bone allows, during bone resorption and the dissolution of hydroxyapatite, the complex to be released and initiate new bone formation by stimulating the proliferation of osteoblasts in the vicinity of the resorption site. Based on the high affinity of hBD-IGFBP towards both IGF-II and hydroxyapatite, this invention thus provides therapeutic agents and compositions which are useful to, inter alia, target IGF-II and/or IGF-I specifically to bone.

The novel hBD-IGFBP, hBD-IGFBP/IGF-II and other conjugates, antibodies to hBD-IGFBP and antagonists thereof, and pharmaceutical compositions prepared therefrom are particularly useful for administration for treatment of a wide variety of hBD-IGFBP and IGF-II related disease. Preferably, the pharmaceutical compositions can be administered parenterally, i.e., subcutaneously, intramuscularly or intravenously, or topically, orally, via aerosol, intranasal delivery and the like. Thus, this invention provides

compositions for parenteral administration which comprise a solution of the hBD-IGFBP, hBD-IGFBP/IGF-II and other conjugates, antibodies to hBD-IGFBP and antagonists thereof or a cocktail of hBD-IGFBP and IGF-II dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine and the like. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, etc. The concentration of the desired hBD-IGFBP, hBD-IGFBP/IGF-II, or antibodies to hBD-IGFBP or other antagonists thereof in these formulations can vary widely, i.e., from less than about 0.00001%, usually at or at least about 0.001%, to as much as about 0.05 to 0.1% by weight and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected, the condition being treated, e.g., fracture repair, osteoporosis, surgical or traumatic wound repair, tumors such as osteosarcoma or breast carcinomas, etc., and the subject being treated, i.e., an adult, child or neonate.

Thus, a typical pharmaceutical composition for intravenous infusion to treat an adult suffering from moderate osteodegenerative disease could be made up to contain 250 ml of sterile Ringer's solution, and about 50 mg to 5 grams of hBD-IGFBP or hBD-IGFBP/IGF-II. Actual methods for preparing parenterally or orally administrable compounds will be known or apparent to those skilled in the art and are described in more detail in for example, Remington's Pharmaceutical Science, 16th ed., Mack Publishing Company, Easton, PA (1982), which is incorporated herein by reference.

The compositions containing the present hBD-IGFBP or hBD-IGFBP/IGF-II or cocktails thereof can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, compositions are administered to a patient

already suffering from an hBD-IGFBP or IGF-II related disease, in an amount sufficient to cure or at least partially arrest the disease and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend on the disease, i.e., bone degeneration as in osteoporosis, fracture, wound, tumor, etc., and its severity, the age of the patient and the general state of the patient's health. Generally, the amounts will range from about 1.0 to about 500 μg of hBD-IGFBP or hBD-IGFBP/IFG-II per kilogram of body weight per hour of infusion, with dosages of from 10 to 50 μg of hBD-IGFBP or hBD-IGFBP/IFG-II per kilogram per hour of infusion being more commonly used. As the materials of the present invention may be employed in serious disease states, in view of the minimization of extraneous substances and the absence of foreign substance responses, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these pharmaceutical compositions.

In prophylactic applications, compositions containing the present hBD-IGFBP or hBD-IGFBP/IFG-II or cocktails thereof are administered to a patient not already in a disease state to enhance the patient's resistance to the disease. Such an amount is defined to be a "prophylactically effective dose." In this use, the precise amounts again depend on the patient's state of health, etc. but generally range from 1 to 500 μg per kilogram per hour of infusion, especially 10 to 50 μg per kilogram per hour. A preferred prophylactic use is for treatment of patients at risk of severe osteodegenerative diseases.

Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulations should provide a quantity of hBD-IGFBP or hBD-IGFBP/IGF-II, for example, sufficient to treat the patient.

In yet another aspect, the invention provides an agent which is effective in stimulating bone cell proliferation in response to IGF-II. Exogenous addition of IGF-II to bone

cells in serum free conditions increases their proliferation. This proliferative effect of IGF-II is potentiated by the addition of hBD-IGFBP in conjunction with IGF-II. This synergistic action of the combination of hBD-IGFBP and IGF-II, that is, greater than the additive effect achieved by combining the results obtained with either agent individually, has not been reported for other IGFBPs in any cell type. Thus the invention provides a therapeutic agent for the treatment of bone disorders (e.g. osteoporosis) where bone formation is impaired. The pharmaceutical compositions will comprise the hBD-IGFBP and, if desired, IGF, with physiologically acceptable carriers and or excipients.

This invention also provides an agent that can be used in general wound healing and in skin repair to increase the potency and half life of IGFs, as hBD-IGFBP may increase the half-life of IGFs by protecting them from proteases, act to target IGFs specifically to bone, and/or potentiate the proliferative action of IGFs.

Complexes of hBD-IGFBP + IGF can be prepared in a variety of ways, e.g., by incubating concentrations of purified hBD-IGFBP and IGF at neutral pH overnight prior to administration. The concentrations of hBD-IGFBP and IGF in the composition can vary widely, but preferably are approximately equimolar. Other formulations will be apparent to the skilled artisan from the context of the present disclosure. When formulated separately, the compositions of hBD-IGFBP can be administered separately or simultaneously with the IGF-II compositions. When administered separately, typically the hBD-IGFBP will be administered first, followed by the IGF-II. Such compositions can be administered in specific areas to stimulate local bone formation (e.g., fracture repair) or administered systemically to increase general bone formation, as in the treatment of bone disorders such as osteoporosis.

In other embodiments, the present invention provides for the preparation of more potent IGF molecules. Structure-function analysis of IGFs and hBD-IGFBP can identify region(s) of IGFs that is involved in binding to hBD-IGFBP. It is possible to produce modified IGF molecules by amino acid

substitutions, insertions or deletions such that the modified molecules bind to IGF receptors and the hBD-IGFBP with high affinity but not to inhibitory IGFBPs, such as IGFBP-4 and IGFBP-3. Such modified IGF molecules can serve as potent anabolic agents in promoting general wound healing and in skin repair. In other embodiments fragments of the hBD-IGFBP are produced. The fragments will typically have a desired function, such as the ability to bind IGF-II or hydroxyapatite, while eliminating other portions of the molecule which are not essential for this function. Fragments may be used individually or joined together.

In addition to the IGFs, the hBD-IGFBP of the invention are useful in targeting other molecules of interest to the bone. The molecules may affect the formation or resorption of bone directly or indirectly. As will be apparent to those of skill in the art, a wide variety of agents can be targeted to bone tissue in this manner. Representative examples include those which stimulate bone formation, such as bone morphogenic protein (BMP), TGF_{β}, fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), agents which decrease bone formation as may be desired in certain cancers, such as glucocorticoid or 1, 25-dihydroxyvitamin D_3, those compounds which increase bone resorption, such as macrophage colony stimulating factor (M-CSF) and interleukins, and compounds which decrease bone resorption, such as bis-phosphonate and calcitonin, for example. The compounds can be joined to the hBD-IGFBP of the invention in a variety of ways, including conjugation means set forth above as well as fusion and chimeric proteins, as appropriate. Typically dosages of the above mentioned targeted compounds such as growth factors will be delivered to the surface of the bone tissue at concentrations ranging from about 10 pg/ml up to about 50 ng/ml of bone tissue.

In another aspect, the invention provides a diagnostic marker to evaluate bone formation in clinical samples taken from patients who have metabolic bone disease or bone neoplasia. Based on the findings that hBD-IGFBP is an important modulator of IGF-II actions and that IGF-II is an

important human bone growth factor, levels of hBD-IGFBP can be used to monitor disorders of bone metabolism, where aberrant levels of hBD-IGFBP represent present of the disorder. Thus the invention also provides reagents for a clinical diagnostic bone formation marker to monitor bone formation during treatment of bone disorders with therapeutic agents. The compositions of hBD-IGFBP or antibodies thereto can be used for the detection and quantitation of hBD-IGFBP in a biological fluid, such as human plasma, serum or urine.

As will be recognized by those skilled in the art, numerous types of immunoassays are available for use in the present invention. For instance, direct and indirect binding assays, competitive assays, sandwich assays, and the like, are generally described in, e.g., U.S. Pat. Nos. 4,642,285; 4,376,110; 4,016,043; 3,879,262; 3,852,157; 3,850,752; 3,839,153; 3,791,932; and Harlow and Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Publications, N.Y. (1988), each incorporated by reference herein. In one assay format hBD-IGFBP is quantified directly by measuring the binding of antibodies to hBD-IGFBP, which antibodies are then detected by, e.g., labeled anti-IgG, IgM and/or IgA human antibodies. In another format, a patient's hBD-IGFBP can be measured by competing with labeled or unlabeled hBD-IGFBP, for binding. A wide variety of labels may be employed, such as radionuclides, particles (e.g., gold, ferritin, magnetic particles, red blood cells), fluors, enzymes, enzyme substrates, enzyme cofactors enzyme inhibitors, ligands (particularly haptens), chemiluminescers, etc., but preferably radionuclides.

Thus, hBD-IGFBP or antibodies thereto for use in such assays can be attached to an insoluble or solid support, such as an ELISA microtiter well, microbeads, filter membrane, insoluble or precipitable soluble polymer, etc. to function as an affinity resin. The antisera or monoclonal antibodies to hBD-IGFBP are typically non-human in origin, such as rabbit, goat, mouse, etc. Kits can also be supplied for use in detecting the hBD-IGFBP, where the hBD-IGFBP and/or antibodies thereto may be provided, usually in lyophilized form, in a

container, either alone or in conjunction with additional reagents, labels, and/or anti-antibodies, and the like. The hBD-IGFBP polypeptide and antibodies, which may be conjugated to a label, or unconjugated, and are included in the kits with buffers, such as Tris, phosphate, carbonate, etc., stabilizers, biocides, inert proteins, e.g., serum, albumin, or the like. Frequently it will be desirable to include an inert extender or excipient to dilute the active ingredients, where the excipient may be present in from about 1 to 99% of the total composition.

Antibodies for diagnostic or therapeutic uses which bind hBD-IGFBP polypeptides of the invention can be produced by a variety of means. The production of non-human monoclonal antibodies, e.g., murine, is well known and may be accomplished by, for example, immunizing the animal with a recombinant or synthetic hBD-IGFBP molecule or a selected portion thereof (e.g., a peptide). For example, by selected screening one can identify a region of the hBD-IGFBP molecule such as that predominantly responsible for recognition by IGF, if desired. Antibody producing cells obtained from the immunized animals are immortalized and screened, or screened first for, e.g., the production of antibody which binds the hBD-IGFBP, and then immortalized.

The following examples are offered by way of illustration, not limitation.

EXAMPLE I

Preparation of human bone extract for purification of hBD-IGFBP

Human femoral heads obtained during total hip replacement surgery were stored frozen at -20°C until used. Bones were cut using a band saw and ground to fine particles in a Wiley mill for hBD-IGFBP extraction. Bone proteins were extracted by demineralization of the femoral head bone powder with 10% ethylenediaminetetraacetate (EDTA) in the presence of 4M guanidine HCl and protease inhibitors (Guanidine EDTA extract) after an initial extraction with water and 4M guanidine HCl as described in Mohan, et al., (Biochem. Biophys. Acta, 884:234-242 (1986)). Guanidine EDTA extract was then

concentrated in an Amicon using YM5 (5 kilodaltons molecular weight cut-off) membrane and used for the purification of hBD-IGFBP.

EXAMPLE II

Purification and characterization of hBD-IGFBP from human bone extract

Human bone extract was subjected to hydroxyapatite (HA) chromatography in 4 M guanidine HCl as described in Mohan, et al., ibid. An IGF-II affinity column was constructed by coupling 250 μg of IGF-II purified from human bone to cyanogen bromide-activated Sepharose 4B beads. A pool of HA bound fractions was concentrated in an Amicon cell using a YM5 membrane to about 300 ml and dialyzed against 20 times volume of potassium phosphate buffer (10 mM potassium phosphate, pH 6.0) containing protease inhibitors (100 mM epsilon aminocaproic acid, 5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride). The IGF-II affinity column was equilibrated with the potassium phosphate buffer after which a 50 ml aliquot (approximately 3-4 mg total protein/ml) of the dialyzed HA bound fraction pool of the human bone extract was loaded into the column. The column was then extensively washed with potassium phosphate buffer to completely remove the unbound proteins. The bound proteins were eluted with 20-25 ml of 30 mM tris-acetate (pH 7.2)/4 M guanidine-HCl. The affinity bound fraction was concentrated in an Amicon cell using a YM 5 membrane and then dialyzed against 20 mM tris-HCl (pH 8.0) buffer. The dialyzed affinity bound fraction was applied to a Pharmacia FPLC Mono Q anion exchange column previously equilibrated with the tris-HCl buffer. The bound proteins were eluted with a linear gradient from 0-1 M NaCl in 20 mM tris-HCl buffer in 100 min.

hBD-IGFBP activity was determined by a polyethylene glycol precipitation method. Briefly, 50 μl of sample to be assayed was incubated with 25,000 to 50,000 cpm of ^{125}I-labeled IGF-I or IGF-II for 60 minutes at room temperature in 250 μl of 0.1M HEPES/0.1% bovine serum albumin/0.1% Triton X100/44 mM Na_2CO3/0.02% NaN3, pH 6.0. To this mixture was added 100 μl of 2% immune serum globulin and 500 microliters of 25%

polyethylene glycol, followed by centrifugation. Under these conditions the polyethylene glycol precipitated the larger complex between IGF-I or IGF-II and hBD-IGFBP, but did not precipitate unbound IGF-I or IGF-II. The amount of ^{125}I-IGF-II in the PEG precipitate was then counted. Non-specific binding was determined by carrying out the assay in the presence of excess unlabeled IGF-I or IGF-II and the amount of ^{125}I-IGF-I or ^{125}I-IGF-II precipitated was subtracted from the value obtained above.

To determine the apparent molecular weight of hBD-IGFBP, ligand blot analysis was carried out using ^{125}I-IGF-II as a tracer. In this procedure, 50 μl of samples was electrophoresed under non-reducing conditions on precast 3-27% SDS polyacrylamide slab gels. After transfer of the samples to nitrocellulose by electroblotting, the nitrocellulose membrane was incubated with radiolabeled IGF-II. After washing the unbound radiolabeled IGF-II, the membrane was subjected to autoradiography as described in Hossenloop, et al., (Anal. Biochem., 154:138-143 (1986)).

Amino acid compositions of samples were analyzed with an Applied Biosystems model 420 analyzer and N-terminal sequences were determined with an Applied Biosystems model 470A vapor phase protein sequencer (Mohan, et al., Biochim. Biophys. Acta, 966:44-55 (1988)).

Fig. 3 shows the protein profile and IGFBP activity profile of the affinity-bound fraction pool in the Mono Q chromatography step. There was neither a protein absorbance peak nor a IGFBP activity peak in the region where authentic IGFBP-4 elutes (Fractions 9-15, 0.1M NaCl), thus suggesting that the bone derived IGFBP is not IGFBP-4. However, there were four protein absorbance peaks eluting at different NaCl concentrations. Of the four protein peaks, the first two peaks (A & B) contained significant IGFBP activity while the last two protein peaks (C & D) had little IGFBP activity.

To examine the apparent molecular weights of the IGFBPs present in human bone extract, ligand blotting and [^{125}I]IGF-II affinity labeling were used. Fig. 4 shows ligand blots in which the HA bound, IGF-II bound and Mono Q protein

peaks were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with [^{125}I]IGF-II tracer. The major IGFBP present in HA bound and IGF-II bound fractions had an apparent molecular weight of 29 kDa. In addition, these fractions also exhibited a broad, less intense band between the 68 and 43 kDa molecular weight markers. The major 29 kDa IGFBP was separated from the higher molecular weight IGFBP by Mono Q chromatography. Mono Q peak A showed a major band at 29 kDa and a minor band at 24 kDa. Mono Q peak B showed a broad diffuse band between the 68 and 43 kDa markers and a minor band at 29 kDa. Mono Q peak C (represents the major protein absorbance peak) and D showed only a weak band at 29 kDa. These data suggest that the major IGFBP in human bone extract is a 29 kDa IGFBP.

Both the amino acid composition and the N-terminal amino acid sequence of the 29 kDa IGFBP in Mono Q peak A appeared to be unique, having limited sequence similarity to other known IGFBPs (Tables 1, 2; Fig. 1).

Table 1. Amino acid compositions of hBD-IGFBP and the known IGFBPs

	Peak A	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4
asx	8.1	6.8	6.6	6.8	8.0
glx	5.9	13.2	13.9	10.2	12.2
ser	9.3	9.0	3.5	10.2	5.9
gly	9.0	7.3	11.8	8.7	9.7
his	5.1	2.6	3.8	2.7	4.6
arg	6.3	4.3	6.9	7.2	8.0
thr	6.3	3.8	3.8	3.4	2.5
ala	7.9	11.1	7.3	6.8	6.8
pro	5.8	7.7	9.3	8.7	8.9
tyr	3.7	2.6	1.7	3.4	1.3
val	6.6	3.8	5.5	5.3	3.8
met	3.5	1.3	3.1	0.8	1.7
cys	5.5	7.7	6.6	6.8	8.4
ile	3.3	3.8	1.4	2.3	2.5
leu	6.8	7.3	9.0	7.2	8.0
phe	2.9	1.7	1.0	1.9	2.1
lys	4.0	3.8	4.5	7.2	5.1
trp	2.1	0.3	0.4	0.4	

10 mm diameter immobilon membrane cut-outs were soaked in methanol, placed into Millipore filtration units, and held in place with rubber O-rings. After rinsing the immobilon membrane with water, the BP in Peak A was fixed into the membrane by filtering through 1 ml of Peak A. One-half of the membrane was used for amino acid composition studies.

Table 2. Aminoterminal sequence of hBD-IGFBP in MonoQ Peak A.

Residue	Amino acid (pmoles)
1)	L=59.4
2)	G=50.1
3)	F=34.8
4)	F=48.0
5)	V=49.1
6)	X
7)	V=27.1
8)	E=20.6
9)	P=22.2
10)	D=15.2
11)	D=18.7
12)	K=13.4
13)	A=22.8
14)	A=32.8
15)	L=28.5

50 pmoles of hBD-IGFBP was used for N-terminal amino acid sequence analysis. Combined average repetitive yield was 86.3%. X = not known.

Therefore, the 29 kDa IGFBP has been designated human bone derived IGFBP (hBD-IGFBP). In addition to the major sequence (Leu-Gly-Phe-Phe-Val-X-Val-Glu-Pro-Asp-Asp-Lys-Ala-Ala-Leu), there was evidence for the presence of an additional sequence in Mono Q peak A which lacked one or two amino acids at the N-terminus. The purified hBD-IGFBP appeared to be susceptible to proteolytic cleavage since storage of purified 29 kDa IGFBP at 5^{o}C for overnight led to the disappearance of 29 kDa band and appearance of a major band at 24 kDa and several low molecular weight minor bands. Upon N-terminal sequence analysis of the 24 kDa band, the sequence obtained (Leu-Gly-Phe-X-Val-X-X-Glu-Pro-X-X-Lys) was similar to the sequence of 29 kDa IGFBP. Further storage of the 24 kDa IGFBP led to its disappearance and appearance of several low molecular weight protein bands which appeared to contain very little IGFBP activity as determined by

ligand blot analysis. Consistent with these results, proteases associated with IGFBPs have been recently reported.

Mono Q peak B appeared to have a different amino acid composition and did not potentiate the action of IGF-II on bone cells. Our attempts to sequence peak B proved unsuccessful. The sequence determination of the first few cycles of the major protein peak (Fraction 45, peak C), yielded multiple amino acids with no readable sequence. Thus, Mono Q peaks C and D which contained very little IGFBP activity may represent the degradation products of the 29 kDa IGFBP.

Since N-terminal sequence of Mono Q purified hBD-IGFBP peak yielded in addition to the major sequence, additional sequences which lacked one or two amino acids (perhaps due to co-purification of IGFBP protease which degrades this IGFBP) and since the cysteine residues were not derivatized, the valine at position 7 of the present hBD-IGFBP sequence and the aspartic acid at position 10 may be cysteines (cysteine residues have been preserved among different members of IGFBP family). Storage of Mono Q purified human bone derived IGFBP at 5°C led to disappearance of the 29 kDa IGFBP and appearance of smaller molecular weight IGFBPs by SDS-PAGE. When the small molecular weight IGFBP was sequenced, the valine and aspartic acid at position 7 and 10 respectively were not found, in that there were no signals at these positions. These findings suggest that hBD-IGFBP may have the sequence of L-G-F-F-V-X-C-E-P-C-D-K-A-A-L in one embodiment. An alternative sequence for hBD-IGFBP which also takes into account the foregoing considerations is: L-G-S-F-V-H-C-E-P-C-D-E-K-A-L, which sequence is similar to the BP-5 sequence of Kiefer et al., Biochem. Biophys. Res. Comm. 176:219-225 (1991), Shimasaki et al., J. Biol. Chem. 266:10646-10653 (1991), and as disclosed in Drop, Endocrinol. 130:1736-1737 (1992). The sequence may be subject to possible variations based on native or introduced substitutions, additions or deletions, which

variations may be allelic variants or produced through particular sequence techniques employed herein. It will be recognized that using the methods described herein, the protein may be isolated and purified and the sequence determined by a variety of well known methods. Further, the N-terminal sequence allows for the construction of degenerate oligonucleotide probes for the cloning of the gene which encodes the hBD-IGFBP of the invention.

EXAMPLE III

Use of hBD-IGFBP in bone cell proliferation assays

An assay for IGF-mediated proliferation of bone cells in serum-free culture medium, which is described in Mohan, et al., (Biochim. Biophys. Acta, 884:234-242 (1986)), hereby incorporated by reference, measures the incorporation of [^{3}H]thymidine into trichloroacetic acid precipitable cellular material. This assay was performed using the mouse osteoblastic cell line MC3T3-E1. Approximately 10,000 cells were plated per well in serum-free Dulbecco's modified Eagle's medium into 48-well culture dishes and used for the [^{3}H]thymidine assay as generally described in Mohan et al., ibid.

In this assay hBD-IGFBP by itself had little mitogenic activity, as determined by the incorporation of [^{3}H]thymidine into trichloroacetic acid insoluble macromolecules (Table 3, below). However, when hBD-IGFBP was added along with submaximal concentrations of IGF-II to serum-free cultures of mouse bone cells, hBD-IGFBP potentiated the proliferative action of IGF-II. IGFBP-3 has been shown to increase IGF-I action only when added to cultures several hours prior to the addition of IGF-I, (Mohan, et al., Proc. Natl. Acad. Sci. USA, 86:8338-8342 (1989) and De Mellow, et al., Biochem. Biophys. Res. Comm., 156:199-204 (1988)) and no other IGFBPs are believed to have been shown to potentiate the actions of IGFs under conditions where both an IGF and an IGFBP are

added simultaneously. These data suggest that the hBD-IGFBP is not merely a passive carrier for the IGFs but also positively regulates the action of the IGFs. Although the mechanism(s) by which hBD-IGFBP potentiates IGF-II stimulated [^{3}H]thymidine incorporation is not known, several mechanisms are offered by way of possible explanation but not limitation. For example, the hBD-IGFBP targets the IGF-II to the cell membrane for easy access of the IGF-II to its receptor (perhaps through an RGD sequence as in the case of IGFBP-1). Or the hBD-IGFBP may increase the affinity of IGF-II to its receptor by virtue of its binding to IGF-II, and/or increase the half-life of IGF-II by protecting it from proteases.

Table 3

Potentiating effect of hBD-IGFBP on IGF-II induced bone cell proliferation

Treatment	[^{3}H]thymidine incorporation (% of control) Experiment 1	Experiment 2	Experiment 3
BSA control	100 ± 15	100 ± 22	100 ± 12
hBD-IGFBP	128 ± 18	213 ± 29	116 ± 9
IGF-II	138 ± 18	265 ± 43	214 ± 24
hBD-IGFBP + IGF-II*	195 ± 32	672 ± 74	320 ± 40

Serum free cultures of mouse osteoblastic cell line, MC3T3-E1 were incubated for 18 hrs with the effectors prior to the addition of [^{3}H]thymidine. The final concentrations of IGF-II and hBD-IGFBP were 3 and 10 ng/ml respectively. Values are Mean ± SD of 6 replicate wells. [^{3}H]thymidine incorporation in bovine serum albumin (BSA) treated control cultures were 1404 ± 217, 237 ± 53 and 730 ± 91 respectively in the three experiments.
* The interaction term between hBD-IGFBP and IGF-II was highly significant ($P<0.00001$) by three way analysis between experiment, hBD-IGFBP and IGF-II using CSS computer program.

EXAMPLE IV

Purification of hBD-IGFBP from bone cell conditioned medium

Since bone cells in culture produce hBD-IGFBP, serum free conditioned medium collected from bone cell cultures can also be used for the purification of hBD-IGFBP. Briefly, bone cell conditioned medium is concentrated in an Amicon using YM5 (5 kilodalton molecular weight cut-off), acidified with acetic acid to a final concentration of 1M and subjected to Sephadex G-100 gel filtration to separate IGFs from IGFBPs. The proteins are eluted with 1M acetic acid. The fractions containing IGFBPs are pooled, lyophilized, reconstituted

with phosphate buffered saline and subjected to an IGF-II affinity column. The affinity bound proteins are then subjected to FPLC Mono Q anion exchange chromatography to separate hBD-IGFBP from other IGFBPs.

EXAMPLE V

Quantitative diagnostic assay for hBD-IGFBP

hBD-IGFBP purified from human bone or expressed by recombinant means is used for polyclonal and/or monoclonal antibody production, which antibodies are then used in quantitative assays for hBD-IGFBP. Briefly, hBD-IGFBP is mixed with complete Freund's adjuvant and injected into rabbits, guinea pigs, rats or mice following established protocols for antibody production. Animals are subsequently injected with hBD-IGFBP mixed with incomplete Freund's adjuvant every 3-4 weeks. For polyclonal antisera, the animals are bled after 3-4 injections and the antibody titer to hBD-IGFBP determined using radioimmunoassay or other means. Monoclonal antibodies are produced by immortalizing antibody producing cells obtained from the immunized animals using well known techniques. Purified hBD-IGFBP is radiolabeled and used as the signal-producing tracer. The monoclonal antibody or antiserum with high titer is then used for development of hBD-IGFBP radioimmunoassay for measurement of hBD-IGFBP levels in the serum urine and other biological fluids.

In general, the production of hBD-IGFBP is increased by treatment of bone cells with agents which increase bone cell proliferation. Thus, hBD-IGFBP can be used as diagnostic marker for disease states associated with bone cell proliferation, such as osteoporosis. Accordingly, low serum hBD-IGFBP indicates osteoporosis associated with low bone formation. Since hBD-IGFBP potentiates IGF-II action, a high serum hBD-IGFBP may also be associated with some cancers.

EXAMPLE VI

Quantitative diagnostic assay for IGFs using hBD-IGFBP

As described in this Example, recombinant or purified hBD-IGFBP can also be used to quantify levels of IGFs in a biological sample, such as serum. 2-5 ng of purified hBD-IGFBP was incubated with 40,000 cpm of ^{125}I-IGF in the presence or absence of unlabeled competitor. IGF standards or sample containing unknown amounts of IGF were used as competitors. After 60 minutes of incubation at room temperature, hBD-IGFBP-IGF complex was precipitated by adding polyethylene glycol in the presence of bovine gamma globulin. After a 30 minute centrifugation at 1185 X g, an aliquot of the supernatant was counted in a gamma counter. A standard curve was set up with different concentrations of unlabeled IGF and the amount of IGF in the unknown sample was calculated using the standard curve. Thus the amount of biologically active free IGFs was determined by this assay utilizing purified hBD-IGFBP that has high affinity for IGFs.

EXAMPLE VII

hBD-IGFBP Fixes IGF-II in bone

Human bone contains a relatively large amount of IGF-II. However, as shown in Table 4, ^{125}I labeled IGF-II by itself does not specifically bind to hydroxyapatite (the Table shows only non-specific binding which is less than 10% of total counts added) or to collagen. In contrast to IGF-II, labeled hBD-IGFBP exhibited specific binding to hydroxyapatite. The binding of hBD-IGFBP to hydroxyapatite was specific since the major serum binding protein, i.e., IGFBP-3 had no similar activity and since hBD-IGFBP did not bind to collagen, the other major constituent of bone. Furthermore, the binding of hBD-IGFBP to hydroxyapatite was strong, in that the hBD-IGFBP-hydroxyapatite complex could not be dissociated with 4M guanidine HCl (4M

guanidine HCl has been shown to dissociate interactions between antibody-antigen complex). Preincubation of labeled IGF-II with hBD-IGFBP prior to the addition to hydroxyapatite column significantly increased IGF-II binding to hydroxyapatite column. This activity of hBD-IGFBP to facilitate the binding of IGF-II to hydroxyapatite was specific in that IGFBP-3 had no such activity. These findings are consistent with a conclusion that under normal conditions IGF-II is fixed in bone by means of hBD-IGFBP.

Table 4: hBD-IGFBP Facilitates the Binding of IGF-II to Hydroxyapatite

Ligand	% Tracer Bound to Hydroxyapatite	Type I Collagen
[^{125}I]IGF-II	<10	<10
[^{125}I]hBD-IGFBP	60	<10
[^{125}I]IGF-II + hBD-IGFBP	45	<10
[^{125}I]IGFBP-3	<10	<10
[^{125}I]IGF-II + IGFBP-3	<10	<10

EXAMPLE VIII

Regulation of IGFBP-5 production in human bone cells in vitro

To determine if human bone cells produce hBD-IGFBP in vitro, Northern blots of total RNA extracted from serum free cultures of MG63, TE85, TE89, SaOs2 and U2 human osteosarcoma cells were hybridized using an oligonucleotide probe against the N-terminal sequence of hBD-IGFBP and using a human IGFBP-5 cDNA probe (Dr. Shimasaki, La Jolla, CA). These studies revealed that all the cell lines tested expressed hBD-IGFBP mRNA. By

Western ligand blot analysis, IGF-I and IGF-II increased production of hBD-IGFBP by several fold in U2 cells. Biological characterization of hBD-IGFBP revealed that this protein potentiated the proliferative action of IGF-II in bone cells.

The proliferation of human osteoblasts and the production of IGF-II stimulated by progesterone in vitro has been reported. In the present experiment the effect of progesterone on the other components in the IGF regulatory system (IGF-I, IGFBPs and IGF receptors) was studied in the human osteoblast-like osteosarcoma cell line MG63. In all experiments, MG63 cells were plated at a density of 7500 cells/cm^2 in DMEM containing 1% calf serum. After overnight incubation, the medium was changed to serum-free before progesterone or vehicle (ethanol) was added. In a time course study, cells were incubated with 100 nM progesterone for 0.5 hours, 2 hours, 4 hours and 6 hours. Northern blot analyses demonstrated increases in mRNA levels for IGF-II, IGF-I, hBD-IGFBP and type-1 and type-2 IGF receptor form 30 minutes to 6 hours compared to respective controls. mRNA levels of the inhibitory IGFBP-4, however, were decreased as early as 30 minutes after progesterone addition. There were no pronounced changes in IGFBP-3 mRNA levels. Thus, progesterone, a steroid hormone which has been shown to stimulate human bone cell proliferation, increases production of IGFBP-5 in human bone cells. The stimulatory effect of progesterone on bone cell proliferation could be mediated not only by increased IGF production, but also by an increase in IGF receptor expression, an increase in hBD-IGFBP (potentiates IGF action), and a decreased production of the inhibitory IGFBP-4.

WHAT IS CLAIMED IS:

1. Purified and isolated human bone derived insulin-like growth factor binding protein, hBD-IGFBP.

2. Purified hBD-IGFBP of about 29 kDal molecular weight which is obtained from human bone or bone cell conditioned media and which binds IGF-II and hydroxyapatite.

3. The purified hBD-IGFBP of claim 2, which potentiates the ability of IGF-II to stimulate proliferation of bone cells.

4. The hBD-IGFBP of claim 2, which has a higher specific binding affinity for IGF-II than for IGF-I.

5. The hBD-IGFBP of claim 2, which binds hydroxyapatite with a binding affinity of at least 10^{-9} M.

6. The purified hBD-IGFBP of claim 2, which is conjugated to a compound that affects bone formation.

7. A pharmaceutical composition which comprises a substantially pure hBD-IGFBP protein of about 29 kDal or a fragment thereof and a pharmaceutically acceptable carrier.

8. The pharmaceutical composition of claim 7, which further comprises IGF-I or IGF-II.

9. The pharmaceutical composition of claim 8, wherein the IGF-I or IGF-II is conjugated to the hBD-IGFBP.

10. The pharmaceutical composition of claim 7, which is formulated for topical application to a patient.

11. The pharmaceutical composition of claim 7, which is formulated for parenteral administration to a patient.

12. A method for modulating the effects of IGF in a patient, comprising administering to the patient a pharmaceutical composition which comprises substantially pure hBD-IGFBP and a pharmaceutically acceptable carrier in an amount sufficient to modulate the effects of the IGF.

13. The method of claim 12, wherein the IGF is IGF-II.

14. The method of claim 13, wherein the hBD-IGFBP is conjugated to IGF-II.

15. The method of claim 12, wherein the patient is suffering from or susceptible to a degenerative bone disorder.

16. The method of claim 15, wherein the bone disorder is osteoporosis.

17. A method for facilitating wound or fracture repair in a patient, which comprises administering to the patient a pharmaceutical composition which comprises substantially pure hBD-IGFBP and a pharmaceutically acceptable carrier in an amount sufficient to facilitate the repair of said wound or fracture.

18. A method for delivering a compound to bone tissue in a patient, which comprises administering to the

patient a pharmaceutical composition which comprises substantially pure hBD-IGFBP or a fragment thereof conjugated to the compound to be delivered and a pharmaceutically acceptable carrier.

19. The method of claim 18, wherein the compound effects bone formation or resorption.

20. A method for determining the presence of hBD-IGFBP in a biological sample, which comprises:

contacting the biological sample with an antibody that specifically binds to hBD-IGFBP under conditions conducive to immune complex formation, and

detecting the presence of immune complex formation between said hBD-IGFBP and said antibody and therefrom determining the presence and/or quantity of hBD-IGFBP in the sample.

21. The method of claim 20, wherein the biological sample is blood, plasma, serum or urine.

22. The method of claim 20, wherein said detecting step is by enzyme reaction, florescence, luminescence, or radioactivity.

23. A method for determining the presence of hBD-IGFBP in a biological sample, which comprises:

contacting under conditions conducive to immune complex formation the biological sample with purified labeled hBD-IGFBP and an antibody that specifically binds to hBD-IGFBP, wherein said antibody is bound to a support, and

detecting the presence of immune complex formation between said labeled hBD-IGFBP and said antibody and therefrom determining the presence and/or quantity of hBD-IGFBP in the sample.

L G F F V X V E P D D K A A L	hBD-IGFBP
A P W Q C A P C S A E K L A	hIGFBP-1
E V L F R C P P C T P E R L A	hIGFBP-2
G A S S G G L G P V V R C E P C D A R A L A	hIGFBP-3
D E A I H C P P C S E E K L A	hIGFBP-4
L A P G X G Q G V Q A G A P G	CEREBROSPINAL FLUID
R A P G C G Q G V Q A G	FIBROBLAST CM
A A P G X G Q G V Q A G C P G	HUMAN SERUM

FIG. 1.

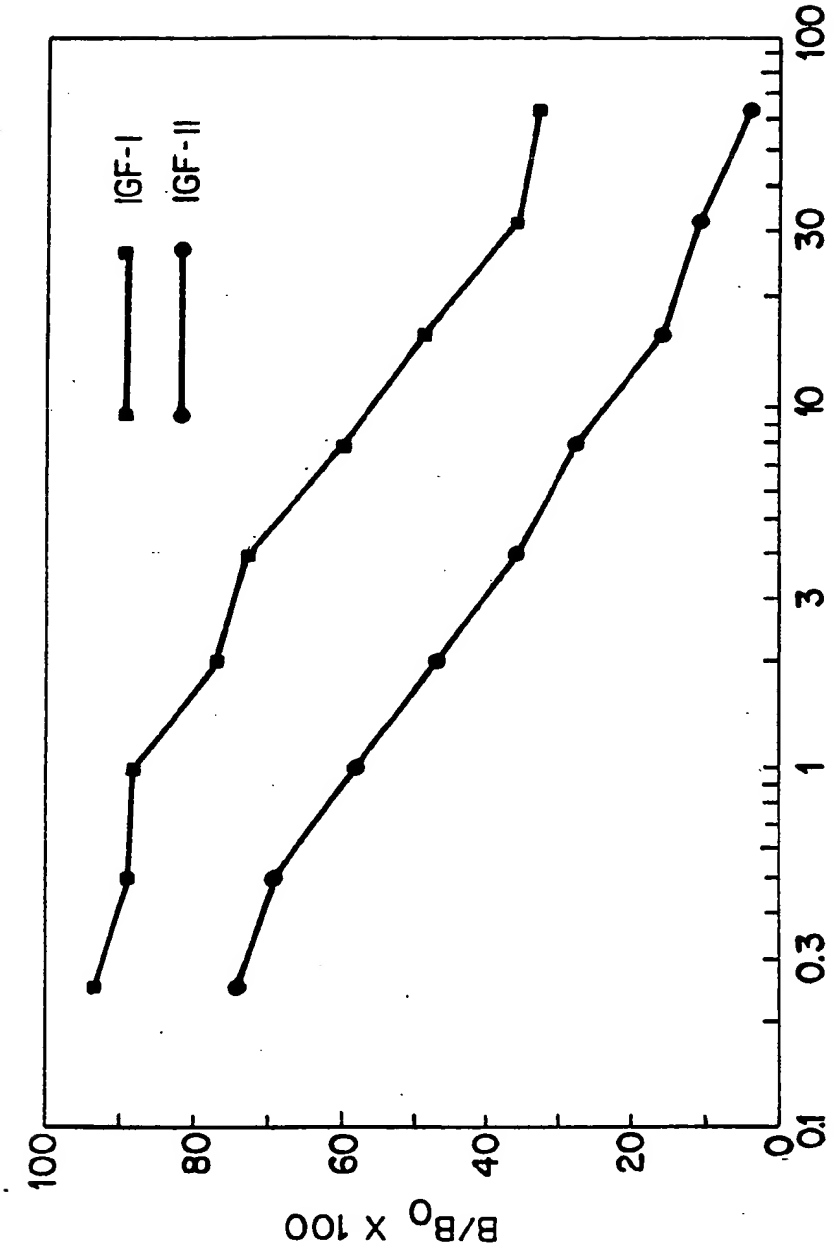
IGF-I
IGF-II
B/B$_O$ X 100
100
80
60
40
20
0
0.1
0.3
1
3
10
30
100
COMPETITOR (ng/ml)

FIG. 2.

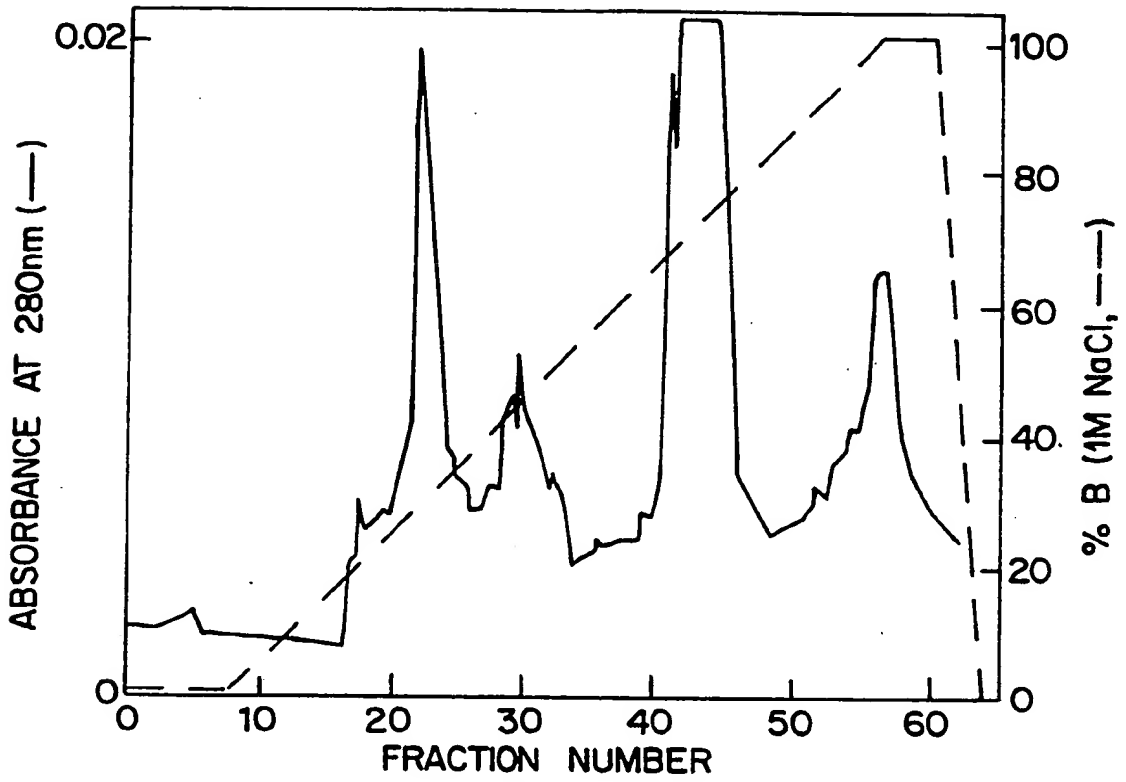
0.02
0
ABSORBANCE AT 280nm (—)
100
80
60
40
20
0
% B (1M NaCl, — —)
0 10 20 30 40 50 60
FRACTION NUMBER

FIG. 3A.

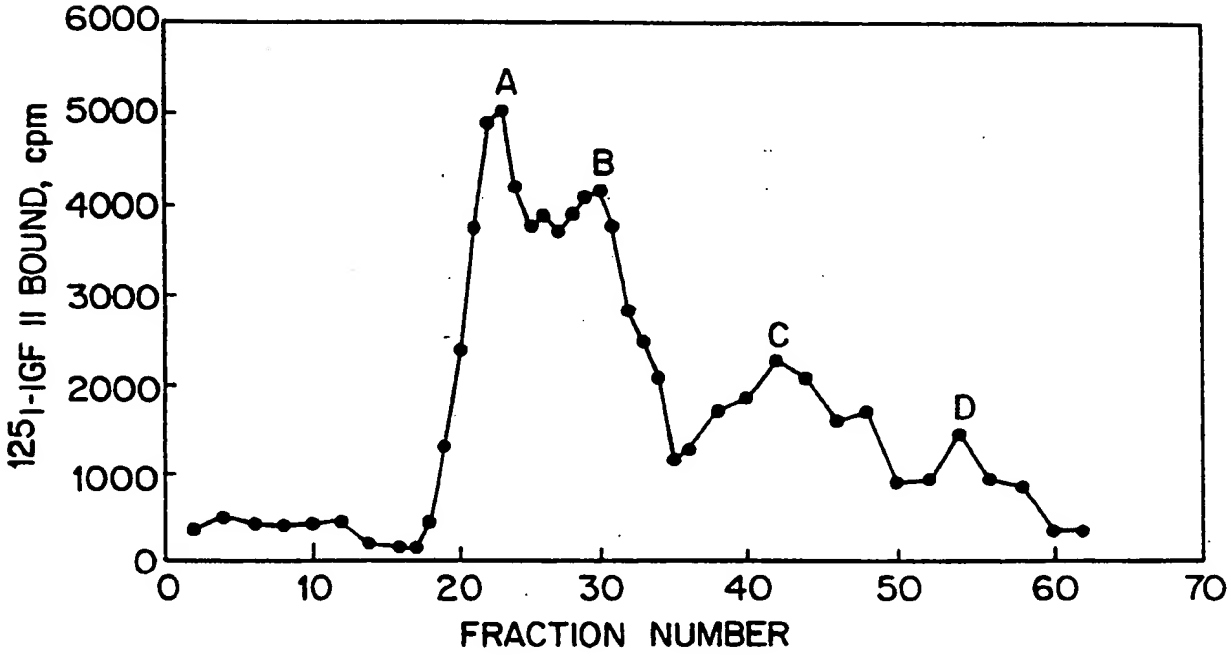
6000
5000
4000
3000
2000
1000
0
^{125}I-IGF II BOUND, cpm
A
B
C
D
0 10 20 30 40 50 60 70
FRACTION NUMBER

FIG. 3B.

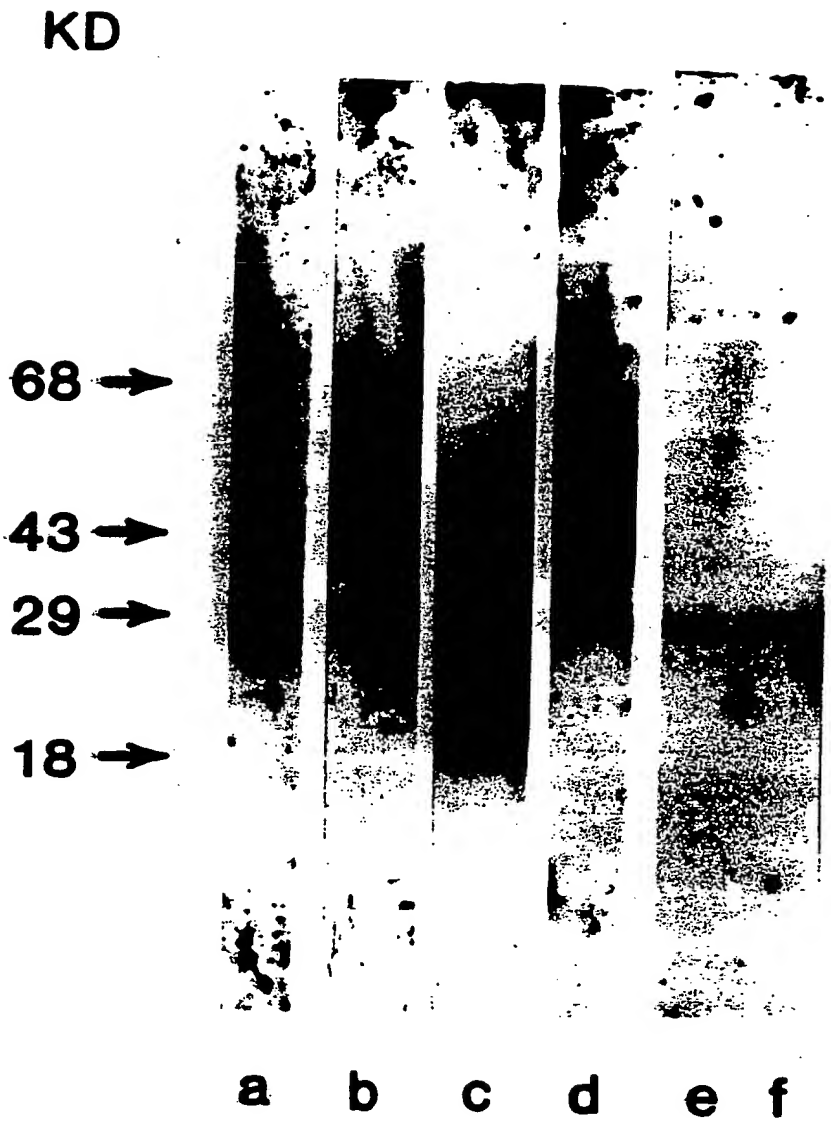

FIG. 4.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/03122

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5) : A61K 37/36, 37/46; C07K 15/06; G01N 33/48
US CL : 530/303, 399; 514/12, 21; 436/63

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	530/303, 399; 514/12, 21; 436/63

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

Cas On-Line and APS

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	EP, A, 0,289,314 (Baylink) 02 November 1988, see page 3, lines 37-46.	2,3,8,9,13, 14
A	US, A, 4,642,120 (Nevo et al) 10 February 1987.	1-23
X/Y	Proc. Natl. Acad. Sci., Volume 86, issued November 1989, S. Mohan et al. "Isolation of an inhibitory insulin-like growth factor (IGF) binding protein from bone cell-conditioned medium; A potential local regulator of IGF action" see the entire document, particularly the abstract.	1-4,10-12 5-9,13-23

* Special categories of cited documents:[16]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
27 JUNE 1992	27 JUL 1992
International Searching Authority[1]	**Signature of Authorized Officer[20]**
ISA/US	FATEMEH T. MOEZIE LG